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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO (If known See 37 CFR 1.5)
INTERNATIONAL APPLICATION NO. PCT/FR99/01326	INTERNATIONAL FILING DATE 04 June 1999	PRIORITY DATE CLAIMED 05 June 1998 US priority--60/122,599 03 March 1999
TITLE OF INVENTION POLYPEPTIDES WHICH POSSESS AN ACTIVITY OF THE B-SECRETASE TYPE		
APPLICANT(S) FOR DO/EO/US Mohamed RHOLAM, Noeli MUNOZ-GIMENEZ, Mohamed MOUTAOUAKIL, Paul COHEN, Phillipe BERTRAND		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<ol style="list-style-type: none"> <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. <input type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). <input checked="" type="checkbox"/> has been transmitted by the International Bureau. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US) <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ol style="list-style-type: none"> <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). <input type="checkbox"/> have been transmitted by the International Bureau. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. <input type="checkbox"/> have not been made and will not be made. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)), and Power of Attorney. <i>unexecuted</i> <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). 		
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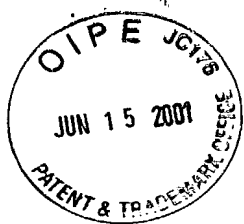
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PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S): RHOLAM ET AL. EXAMINER : UNASSIGNED
SERIAL NO. : 09/701,945 ART UNIT : UNASSIGNED
FILED : December 5, 2000
FOR : POLYPEPTIDES WHICH POSSESS AN ACTIVITY OF THE β -
SECRETASE TYPE

CERTIFICATE OF MAILING UNDER 37 CFR 1.8

I hereby certify that this correspondence is being deposited with the United States Postal Service via First Class mail in an envelope addressed to the COMMISSIONER FOR PATENTS, WASHINGTON, DC 20231 on June 13, 2001.

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(Signature and Date)

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

Dear Sir:

Please amend the above-identified Application as follows:

IN THE SPECIFICATION:

Page 1, line 3, please insert the following:

--PRIORITY CLAIM

This application is a 35 U.S.C. § 371 filing of PCT Application number PCT/FR99/01326 filed on June 4, 1999, which claims the benefit of French Application number 9807068 dated June 5, 1998 and U.S. Provisional Application

number 60/122,599, dated March 3, 1999.--

REMARKS

The above-identified application has been amended to include a Claim of priority.

Thus, no new matter has been added.

Fees

No fees are believed to be necessitated by the instant response. However, should this be in error, authorization is hereby given to charge Deposit Account no. 18-1982 for any underpayment, or to credit any overpayments.

CONCLUSION

Early and favorable action on the claims is earnestly solicited.

Respectfully submitted,



William C. Coppola
Attorney for Applicant(s)
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June 13, 2001

POLYPEPTIDES WHICH POSSESS AN ACTIVITY OF THE β -SECRETASE
TYPE

TECHNICAL FIELD

The present invention relates to novel polypeptides and their pharmaceutical use. More particularly, the present invention relates to novel polypeptides which possess an activity of the β -secretase type and which are characterized in that they are able to cleave the natural β -amyloid peptide precursor (APP) specifically.

BACKGROUND OF THE INVENTION

Individuals who are suffering from Alzheimer's disease exhibit characteristic symptoms of memory distortion and loss of intellectual capacity and cognitive functions. These pathological changes are accompanied by neuronal atrophy, a substantial depletion in a particular type of receptor and also a reduction in synaptic connections. This syndrome involves the presence of a very substantial quantity of senile plaques and neurofibrillary degeneration, principally in the cerebral cortex, the hippocampus, the amygdaloid nucleus and the blood vessels of the cortex.

The so-called senile plaques are spherical structures which become slowly established over ten or so years in the extracellular spaces of the hippocampus, the cortex and other regions of the cerebrum. Their major

constituent is the β -amyloid peptide (β A), which is associated with other abnormal proteins. These structures are surrounded by abnormal axons and neurones.

The neurofibrillary degeneration is due to an accumulation of dense tracts of abnormal fibers in the cytoplasm of particular neurones, principally the pyramidal cells of the cortex. These neurofibrillary tangles consist of a particular form of the tau protein which, when associated with other proteins, gives rise to pairs of helicoidal neurofilaments which disrupt conduction of the nerve impulse.

Familial forms of this disease have been listed and appear to result from various genetic changes, all of which give rise to an abnormal accumulation of the β A peptide. These latter changes, which are very heterogeneous, have, in particular, been linked to various mutations on chromosomes 1, 14 and 21. This latter chromosome has aroused all the more interest because it carries the gene which encodes the β A precursor protein. It can be understood, therefore, why Alzheimer's disease appears at an early age (55) in individuals suffering from Down's syndrome (trisomy 21).

It is to be noted that individuals suffering from familial forms of the disease only constitute a low percentage of all those affected.

In almost all cases of Alzheimer's disease which are not linked to familial forms, those individuals who are more than 70 years old exhibit senile plaques in various regions of the brain. On the other hand, distribution of the plaques differs depending on the type of dementia concerned.

The human β A peptide, which has a molecular mass of 4 kDa, is generated by proteolytic cleavages of its precursor (APP) at the Met⁵⁹⁶-Asp⁵⁹⁷ and

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Val⁶³⁶-Ile⁶³⁷ sites. The liberated molecule consists of 39 (to 42) amino acids having the following protein sequence:

DAEFRHDSGY¹⁰ EVHHQKLVFF²⁰ AEDVGSNKG³⁰ IIGLMVGGVV⁴⁰ IA (SEQ ID #1)

In aqueous solution, this peptide adopts a three-dimensional arrangement of the β -pleated sheet type. Its very hydrophobic COOH-terminal moiety confers on it aggregation properties, with the degree of oligomerization being a function of the pH (maximum formation at pH=5.4) and of the concentration of the peptide. In addition, the sequence between the Gly²⁵ and Met³⁵ residues confers neurotrophic and neurotoxic properties on this peptide.

The β A peptide is a natural product which is secreted by the cells and which can be detected in the blood and the cerebrospinal fluid. Although this peptide is neurotoxic, it is not, however, produced in sufficient quantities to form amyloid plaques. It is thought that an altered processing, or an overexpression, of its precursor predispose to the β A being deposited in the brain.

The primary transcript of the β -amyloid peptide precursor (APP) undergoes alternative splicing to generate mRNAs which encode at least 5 isoforms of 563, 695, 714, 751 and 770 amino acids (a.a.), which isoforms are expressed ubiquitously in the tissues, with their levels differing depending on the cell type.

However, the APP695 and 751 isoforms are exclusively restricted to the central and peripheral nervous system (in particular within the synapses in the astrocytes and the neurones), where they can play a role in the physiological

The 3rd protease activity, termed γ -secretase activity, could also act between the Val⁶³⁶ to Ile⁶³⁷ residues of the precursor in order to generate a secreted proform, APP _{γ} , which contains β A.

The major constituent of the senile plaques which appear both in the familial forms and in the non-familial forms of Alzheimer's disease is the β -amyloid peptide (β A).

The β A peptide results from the cleavage of its precursor, i.e. APP, at the Met⁵⁹⁶-Asp⁵⁹⁷ site of APP, in accordance with a protease activity of the β -secretase type, and at the Val⁶³⁶-Ile⁶³⁷ site, in accordance with a protease activity of the γ -secretase type.

One mutation related to the β -secretase cleavage site has been identified among the familial forms of Alzheimer's disease. This mutation is the double "Swedish" mutation of APP (Lys⁵⁹⁵-Met⁵⁹⁶ \Rightarrow Asn-Leu in APP695), which results in an increased production of the β A peptide (and therefore an increase in the maturation of APP in favor of the amyloidogenic pathway).

However, the fact still remains that, in the very great majority of cases of Alzheimer's disease, the APP is in its natural form with an unmutated β -secretase cleavage site.

Certain proteases derived from man, rats or monkeys have been studied by various authors and are assumed to be involved in the maturation of the APP precursor. Of these enzymes, those which may very particularly be mentioned are the serine proteases 1 and 2 (Abraham et al. (1991), Biochem. Biophys. Res. Commun., 174, 790-796; Matsumoto et al. (1994), Biochemistry, 33, 3941-3948;

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Matsumoto et al. (1994), Neurosciences Letters, 195, 171-174) and the cathepsin G-like protease (Razzaboni et al. (1992), Brain Research, 589, 207-216). While these enzymes of human or simian origin cleave within the Met⁵⁹⁶-Asp⁵⁹⁷ site of APP in accordance with a protease activity of the β -secretase type, they have been detected in, or partially purified from, patients suffering from Alzheimer's disease.

Given that the formation of the β -amyloid peptide results from the action of an enzyme of the β -secretase type on APP, it is easy to understand the importance of identifying and characterizing (an) enzyme system(s) of the β -secretase type which is/are selectively responsible for the post-translational maturation of the β -amyloid peptide precursor at the Met⁵⁹⁶-Asp⁵⁹⁷ site in human cells which are not derived from patients suffering from Alzheimer's disease. Knowledge of these novel enzyme systems thus makes it possible to envisage preparing novel molecules which can be used pharmaceutically and which are, in particular, able to intervene in the metabolism of the β -amyloid peptide in non-familial forms of Alzheimer's disease.

SUMMARY OF THE INVENTION

The present invention provides the identification and characterization of polypeptides which possess a catalytic activity of the β -secretase type with regard to the β -amyloid peptide precursor (APP). Contrary to the other identified proteases, the polypeptides of the present invention have a specificity of action towards the natural form of APP. The present invention ensues, in particular, from demonstrating a 70 kDa polypeptide which is able to

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cleave non-mutated forms of APP. The present invention also provides a process for enzymatically purifying the novel polypeptides, novel cell lines, and novel polyclonal and monoclonal antibodies directed against the polypeptides of the present invention. In addition, the present invention provides a process for identifying compounds which partially or completely inhibit the interaction of the novel polypeptides and the β -amyloid peptide precursor and/or to modulate or inhibit the β -secretase activity of the novel polypeptides. Novel pharmaceutical compositions are also provided which contain as the active ingredient, a polypeptide, an antibody or antisense nucleotide of the present invention.

Figure legends

Figure 1: Topography and cleavage sites of APP.

Figure 2: Description of the process for purifying the polypeptides of the invention.

Figure 3: Immunoblot analysis of the cleavage, by the polypeptides of the invention, of the complete "normal" (APP-K⁵⁹⁵ M⁵⁹⁶) and "double mutated" (APP-N⁵⁹⁵ L⁵⁹⁶) β -amyloid peptide precursors of membrane origin. Demonstration of the cleavage specificity of the polypeptides of the invention towards the "normal" (APP-KM) β -amyloid peptide precursor of membrane origin.

For each of the precursors (APP-NL and APP-KM), track 1 depicts the unincubated membranes without enzyme, track 2 depicts the membranes incubated at 37EC without enzyme, while track 3 corresponds to the membranes incubated at 37EC together with the polypeptides of the invention exhibiting an activity of the β -secretase type.

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DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention relates to polypeptides, or their variants, which possess an activity of the β -secretase type and which are characterized in that they are able to cleave the natural β -amyloid peptide precursor (APP) specifically.

Within the meaning of the present invention, the term "variant" denotes any molecule which possesses the same activity as the polypeptides of the invention and which is obtained by genetic and/or chemical modification of the peptide sequence. Genetic and/or chemical modification may be understood as meaning any mutation, substitution, deletion, addition and/or modification of one or more residues. Such variants can be generated for different purposes, such as that of improving its levels of production, that of increasing its resistance to proteases, that of increasing and/or modifying its activity, or that of conferring on it novel biological properties. Variants resulting from an addition which may, for example, be mentioned are chimeric polypeptides which contain an additional end-linked heterologous moiety. The term variant also comprises polypeptides which are homologous to the polypeptides described in the present invention and which are derived from other cell sources, in particular cells of other organisms.

The substrate which is cleaved by the polypeptides of the invention does not exhibit any mutation in its peptide sequence, and, in particular, the β -amyloid peptide precursor does not carry the double Swedish mutation. The polypeptides of the invention, or their variants, are able to selectively cleave the peptide bond of the Met⁵⁹⁶-Asp⁵⁹⁷ doublet within the native or natural form of

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APP. In particular, the polypeptides of the invention do not cleave the forms of APP which possess the Swedish mutation (Lys⁵⁹⁵-Met⁵⁹⁶ ⇒ Asn-Leu), with this latter fact having been demonstrated on samples obtained from the brains of patients suffering from Alzheimer's disease.

The polypeptides according to the invention were purified from human cells from individuals who were not suffering from Alzheimer's disease and are able to cleave exclusively the natural form of APP within the peptide bond of the Met⁵⁹⁶-Asp⁵⁹⁷ doublet.

The polypeptides of the invention are characterized in that their activity does not depend on a second substrate and/or a ligand. Examples of the latter which may be mentioned are ions, more specifically cations such as magnesium cations or calcium cations. Thus, other proteins, such as serine proteases 1 and 2 or cathepsin G-like protease, possessing a protease activity require the presence of calcium in order to be active.

The polypeptides according to the invention possess a molecular mass of between 65 and 75 kDa, and their molecular mass is preferably about 70 kDa. Their isoelectric point is between 6.0 and 7.0, and is preferably equal to 6.0.

These polypeptides are endopeptidases of the serine protease family. Preferably, these endopeptidases are of the chymotrypsin-sensitive type. Thus, the inhibition profile shows that these endopeptidases are totally inhibited by PMSF (phenylmethylsulfonyl fluoride), and partially inhibited by pefablock,

TPCK(L-1-chloro-3-[4tosylamido]-4-phenyl-2-butanone) and benzamidine.

Furthermore, they are totally resistant to inhibition with antipapain.

The polypeptides of the invention, or their variants, are characterized by having a maximum β -secretase activity at a pH of between 7 and 8.

The invention also relates to non-peptide compounds, or compounds which are not exclusively peptide in nature, which compounds are able to cleave the β -amyloid peptide precursor at the Met⁵⁹⁶-Asp⁵⁹⁷ site. Such compounds are obtained by duplicating the active motifs of the polypeptide according to the invention with non-peptide structures, or structures which are not exclusively peptide in nature, which are compatible with a pharmaceutical use. In this regard, the invention relates to the use of polypeptides as described above for preparing non-peptide molecules, or molecules which are not exclusively peptide in nature, which are active pharmacologically, by determining the structural elements of these polypeptides which are important for their activity and duplicating these elements with non-peptide structures or structures which are not exclusively peptide in nature. The invention also relates to pharmaceutical compositions which comprise one or more molecules which have been prepared in this way.

According to a variant of the invention, the polypeptides, or their variants, additionally comprise a signal sequence which enables them to be located precisely in the cell. Of the sequences which can be used, those which may be mentioned as being preferred are the sequence of the signal peptide of IgkB, the

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signal peptide of APP, the signal peptides of the subunits of the muscle and central nervous system nicotinic acetylcholine receptors, etc.

The invention also relates to a process for enzymically purifying the polypeptides of the invention, which polypeptides possess an activity of the β -secretase type and are able to specifically cleave the natural precursor of APP. This process comprises the following steps:

- the supernatant from the cell culture is first of all concentrated on membranes.
- the concentration product then undergoes the various steps of the purification including, in particular, a step of tangential membrane centrifugation, followed by a step of exclusion chromatography and a step of ion exchange chromatography, then a step of hydrophobic interaction chromatography and, finally, a further step of exclusion chromatography.

The present invention also relates to the use of a cell line. This cell line was selected from a large number of other human cell lines (see Examples) which, while being of diverse origin, derive from individuals who are not suffering from Alzheimer's disease. These cell lines were used to look for polypeptides of the invention or their variants. Thus, these human cell lines represent the central or peripheral nervous system and the immune system and are able to carry out the normal metabolism of the β -amyloid peptide precursor which leads to the latter being produced. The cell line selected is preferably the monocyte-derived THP1 cell line (ATCC TIB 202).

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The cell lines described above are used in particular as hosts for detecting compounds (ligands, antagonists or agonists) capable of inhibiting the interaction between the polypeptides of the invention and their substrate.

The invention also relates to polyclonal or monoclonal antibodies or antibody fragments which are directed against a polypeptide as defined above. These antibodies can be generated using methods known to the skilled person. In particular, these antibodies can be prepared by immunizing an animal against a polypeptide of the invention, or of one of its variants, and then withdrawing the blood and isolating the antibodies. These antibodies can also be generated by preparing hybridomas in accordance with the techniques known to the skilled person. The antibodies or antibody fragments according to the invention can be used, in particular, for their ability to at least partially inhibit the interaction between the said polypeptide and the β -amyloid peptide precursor and/or for at least partially inhibiting the β -secretase activity of the polypeptides of the invention with regard to the natural β -amyloid peptide precursor. In particular, these antibodies are used as medicinal products, especially for treating neurodegenerative diseases such as Alzheimer's disease.

The present invention also relates to a process for identifying compounds which are able to at least partially inhibit the interaction of the polypeptide and the β -amyloid peptide precursor and/or to at least partially modulate or inhibit the β -secretase activity of the polypeptides of the invention.

These compounds are detected and/or isolated in accordance with the following steps:

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- a molecule or a mixture containing different molecules, which may not have been identified, is brought into contact with a recombinant cell such as expressing a polypeptide of the invention under conditions which would enable the said polypeptide and the said molecule to interact if the latter possessed an affinity for the said polypeptide, and

- the molecules which are bound to the said polypeptide of the invention are detected and/or isolated.

In a particular embodiment, this process of the invention is adapted to detecting and/or isolating agonists and antagonists of the β -secretase activity of the polypeptides of the invention. Based on these agonist or antagonist molecules, it is possible to use standard techniques known to the skilled person, in particular sequencing, to obtain their corresponding nucleotide sequences.

Thus, according to one variant of the invention, it can be particularly advantageous to express molecules which are agonists or antagonists of the polypeptides of the invention in situ from their nucleotide sequences. The preparation of these molecules, and their expression in vivo, ex-vivo, and/or in vitro, require their nucleotide sequences to be carried by a viral or plasmid vector and to be transfected, by means of the said vector, into appropriate host cells.

The present invention also relates to the use of the previously defined polypeptides, or their variants, for detecting ligands and compounds which are able to at least partially inhibit the interaction between the polypeptide and the β -amyloid peptide precursor and/or inhibit the β -secretase activity of the polypeptides of the invention or of their variants and/or intervene in the

metabolism of the natural β -amyloid peptide precursor and/or retard production of the β -amyloid peptide.

Thus, the present invention also relates to a method for detecting molecules which are able to influence the activity of the polypeptides of the invention.

This screening method comprises the following steps:

- the polypeptides of the invention, which exhibit an activity of the β -secretase type, are brought into contact with a molecule or a mixture which contains different molecules which may not have been identified.
- the reaction mixture described in the preceding step is brought into contact with the substrate of the polypeptides of the invention, which substrate is preferably APP in its natural form,
- the β -secretase activity on the APP is measured,
- the molecules which had an effect on the β -secretase activity of the polypeptides of the invention are detected and/or isolated.

The invention also relates to the use of a ligand or a modulator, which has been identified and/or obtained using the above-described process, as a medicament. Thus, such ligands or modulators can, by virtue of their ability to interfere with the β -secretase activity level of the polypeptides of the invention with regard to the natural β -amyloid peptide precursor, make it possible to treat certain neurological ailments, in particular Alzheimer's disease.

The invention also relates to any pharmaceutical composition which comprises, as the active principle, either a polypeptide as defined above or the previously defined agonist and antagonist molecules or ligands.

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It also relates to any pharmaceutical composition which comprises, as the active principle, at least one antibody or one antibody fragment as defined above and/or an antisense oligonucleotide.

Furthermore, it also relates to the pharmaceutical compositions in which the above-defined peptides, antibodies, ligands and/or nucleotide sequences are combined with each other or with other active principles.

The pharmaceutical compositions according to the invention can be used for at least partially inhibiting the interaction of the polypeptides of the invention, or of their variants, with the natural β -amyloid peptide precursor and/or for at least partially inhibiting the β -secretase activity and/or intervening in the metabolism of the β -amyloid peptide precursor for the purpose of inhibiting or retarding production of the β -amyloid peptide. The pharmaceutical compositions are more preferably pharmaceutical compositions which are intended for treating neurodegenerative diseases such as Alzheimer's disease.

The present invention also relates to the use of the previously described molecules (ligands, antibodies or antibody fragments, antagonists and agonists) for at least partially inhibiting the interaction of the polypeptides of the invention, or of their variants, and the natural β -amyloid peptide precursor and/or for at least partially inhibiting the β -secretase activity of the polypeptides of the invention, or of their variants, and/or intervening in the metabolism of the β -amyloid peptide precursor for the purpose of inhibiting or retarding production of the β -amyloid peptide. The use of these molecules is preferably envisaged as a medicinal product, especially for treating neurodegenerative diseases and in particular for treating Alzheimer's disease.

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According to one variant of the invention, the polypeptides of the invention, or their variants, are used for intervening in the metabolism of the β -amyloid peptide.

According to another embodiment of the invention, the above-defined polypeptides or their variants are used for detecting ligands or compounds which are able to at least partially inhibit the interaction between the polypeptides of the invention, or their variants, and the natural β -amyloid peptide precursor and/or for at least partially inhibiting the β -secretase activity of the polypeptides of the invention, or of their variants, and/or intervening in the metabolism of the β -amyloid peptide precursor for the purpose of inhibiting or retarding production of the β -amyloid peptide.

For their use according to the present invention, the polypeptides of the invention and, in particular, their antagonists, agonists, antibodies and ligands, are preferably combined with one or more excipients which is/are pharmaceutically acceptable for being formulated with a view to administration by the topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular, transdermal, etc. route. They are preferably employed in an injectable form. The injectable forms can, in particular, be sterile, isotonic saline (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride, etc., or mixtures of such salts) solutions, or dry, in particular lyophilized compositions which, by the addition of sterilized water or of physiological saline, as the case may be, enable injectable solutions to be constituted.

The present invention will be explained in more detail with the aid of the following examples, which are to be regarded as being descriptive and not limiting.

EXAMPLES

Origin of the cell lines

13 human cell lines of varied origin were used to look for maturation enzymes:

Central nervous system

SW 1088	ATCC HTB 12	Astrocytoma
SW 1788	ATCC HTB 13	Astrocytoma
U-138 MG	ATCC HTB 16	Glioblastoma
U-373 MG	ATCC HTB 17	Glioblastoma, astrocytoma, grade III

Peripheral nervous system

HMCB	ATCC CRL 9607	Bowes melanoma
Hs27	ATCC CRL 1634	Newborn foreskin
MRC5	ATCC CCL 171	Lung, diploid

Immune system

DAKIKI	ATCC TIB 206	B-cell, Ig A-secreting
H9	ATCC HTB 176	T-cell lymphoma
IM-9	ATCC CCL 159	Lymphoblast, Ig-secreting
K-562	ATCC CCL 243	Chronic myelogenous leukemia

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RPMI 1788

ATCC CCL 156 Peripheral
blood, IgM-secreting

THP1

ATCC TIB 202

Monocyte

Cell culture

After thawing, the cells are cultured, depending on their origin, either in DMEM medium or in RPMI 1640 medium in the presence of 10% fetal calf serum. These cultures were carried out at 37°C in 1 liter flasks with the culture media being renewed every 2 to 3 days. Depending on the cell line studied, a period of from 2 to 5 months is required in order to obtain an 18 liter volume of culture medium. The last culture step is carried out over 48 hours in the absence of fetal calf serum and phenol red. These cell cultures are then centrifuged in order to recover the supernatant, which is used for purifying the enzymes.

The cell lines HMCB, U-373 MG, U-138 MG, MRC5 and Hs27 were cultured in DMEM medium while the cell lines SW 1088, SW 1783, K-562, H9, DAKIKI, THP1, RPMI 1788 and IM-9 were cultured in RPMI 1640 medium.

Enzyme purification

The 18 liters of supernatant from each cell culture are concentrated on ULTRASETTE™ (FILTRON) membranes having a cut-off threshold of 10 kDa, and the resulting concentration product was then used for purifying the proteolytic activities in accordance with the following protocol:

- The first step consists in centrifuging at 7000 rpm on a tangential membrane.

More specifically, the concentration is effected on an ULTRAFREE7

(MILLIPORE) membrane having a cut-off threshold of 10 kDa

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- A step of exclusion chromatography is then carried out. In accordance with one particular embodiment of the invention, the exclusion chromatography was carried out on a Sephacryl S-100 (Pharmacia) column whose exclusion limits are 10^3 Da and 10^5 Da.

- A step of ion exchange chromatography represents the third step of the process. For this, use was made, in particular, of a Q-Sepharose (Pharmacia) column whose gel consists of strong anions. The column is eluted with a 0 to 1 M saline gradient using solvent A (25 mM Tris, pH 7.5) and solvent B (25 mM Tris, 1 M NaCl, pH 7.5).

- The penultimate step consists of a step of hydrophobic interaction chromatography, in particular on a phenyl-Sepharose 6 (Pharmacia) column having a high degree of substitution ($40 \mu\text{mol/g}$ of gel). This column was eluted with a gradient of from 1 to 0 M ammonium sulfate using solvent A (25 mM Tris, 1 M $(\text{NH}_4)_2\text{SO}_4$, pH 7.5) and solvent B (25 mM Tris, pH 7.5).

- Finally, the last step is a step of exclusion chromatography, which is carried out, in particular, on a TSKgel G2000SW (Interchim) column whose gel consists of rigid silica supports which are grafted with a hydrophilic group. The eluant is a 25 mM Tris buffer, pH 7.5, containing 250 mM NaCl.

Enzyme tests

The β -secretase activities were monitored by means of tests which used various peptides which mimicked or duplicated the amino acid sequence of the APP precursor at the level of the β -secretase-type enzyme cleavage site (Table 1).

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In order to produce the chromophore peptide, 5 μ l of peptide Z-Val-Lys-Met-MCA (7-amino-6-methylcoumarin), diluted 1/1000, are incubated with 5 μ l of supernatant at 37EC for 6 hours. The reaction is stopped by adding 3 μ l of 0.1N HCl and the enzyme activity is determined by measuring the fluorescence of the free AMC chromophore at 460 nm.

Synthetic peptides, which were of different sizes and which mimicked or duplicated the β -secretase-type enzyme cleavage site were synthesized in order to be used as substrates in studying the characterization and specificity of the enzymes (Table 1).

5 μ l of supernatant are incubated with 5 μ l of peptide at 37EC for 6 hours. The reaction is stopped by adding 3 μ l of 0.1N HCl and the enzymic activity is determined by measuring the optical density, at 215 nm, of the cleavage fragments, which have been previously separated by HPLC.

The cleavage sites are deduced by determining the sequence of the fragments resulting from the cleavage.

The percent cleavage [$\% = 100(A_0 - A_j)/A_0$] of each peptide substrate was evaluated by measuring the absorbance at 215 nm (A) of the substrate when incubated in the absence (A_0) and in the presence (A_j) of the enzyme under identical experimental conditions (incubation time, pH and concentration).

The percent inhibition [$\% = 100(I_0 - I_j)/I_0$] of each substrate incubated in the presence of the enzyme was evaluated by measuring the absorbance at 215 nm, in the case of a peptide substrate, or the fluorescence at 460 nm, in the

Taking the 13 human cell lines described in Materials and Methods as the starting material, the search for enzyme activities was carried out using the monoclonal antibody 22C11 for selecting the cell lines which had the ability to produce measurable quantities of APP at the level of the membrane and in the cell

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culture medium. The monoclonal antibody WO-2 was used to reveal and identify the various sites at which the APP was cleaved.

The following results were obtained:

- Use of the monoclonal antibody 22C11 enabled 8 cell lines (HMCB, MRC5, Hs27, SW1088, SW1783, H9, THP1 and IM-9) to be selected out of the total of 13 tested. In the case of the cells which were selected, immunoblot analysis also demonstrated a difference in molecular mass between the membrane APP (120 kDa) and the soluble APPs (110-100 kDa). This indicates that the COOH-terminal sequence of the precursor underwent one or more enzyme cleavage(s).

- immunoblot analysis, using the monoclonal antibody WO-2, of the molecular entities generated in the 8 cell lines selected made it possible to reveal and identify the different sites at which the APP was cleaved and to show that the β A peptide precursor underwent differential maturation.

Thus, this approach made it possible to select cell lines which have the ability to produce measurable quantities of APP at the level of the membrane and in the cell culture medium and to demonstrate enzyme cleavages in the β A peptide precursor.

Peptide substrates:

The peptides [KMD]APP(-5,+5) and Z-Val-Lys-Met-MCA which are derived from APP and which mimic the cleavage site, were used as substrates for detecting the different enzyme activities present in the 8 cell lines.

A combined analysis (HPLC, amino acid composition and sequence determination) of the fragments generated by cleaving the substrate [KMD]APP(-5,+5) was carried out on the cell lines selected. Thus, after incubating the substrate [KMD]APP(-5,+5) with the supernatants, the fragments which were generated were first of all separated by HPLC on a reverse-phase RPC_{18} (VYDAC) column, which was eluted with a 5-40% acetonitrile/0.05% TFA gradient. The sequences and/or the amino acid compositions of these fragments were determined using standard techniques.

The results of this analysis made it possible to identify a predominant cleavage of the Met⁻¹-Asp⁺¹ peptide bond (corresponding to the Met⁵⁹⁶-Asp⁵⁹⁷ site in the intact APP) and 2 minor cleavages of the Ser⁻⁵-Glu⁻⁴ bond (corresponding to the Ser⁵⁹²-Glu⁵⁹³ site in the intact APP) and the Ala⁺²-Glu⁺³ bond (corresponding to the Ala⁵⁹⁸-Glu⁵⁹⁹ site in the intact APP) in each of the 8 cell lines selected.

The inhibition profiles of the enzyme activities of the 8 cell lines with regard to the fluorescent substrate Z-Val-Lys-Met-MCA were also analyzed (Table 2).

The results of this latter analysis revealed the existence of major enzyme activities of the serine (inhibition by aprotinin and pefabloc) and metalloprotease (inhibition by EDTA and phosphoramidon) type in each of the 8 cell lines selected (Table 2).

Example 2. Purification and characterization of the β -secretase activity

The aim of this example is to describe the purification of the polypeptides of the invention possessing a β -secretase activity and to demonstrate their characteristics.

Based on the selection of the 8 human cell lines and the results obtained in Example 1, the cell line THP-1 was chosen, on account of its rapid cell cycle, making it possible to obtain large quantities of protein, for use as a model for purifying the sought-after β -secretase activity in accordance with the purification protocol described in "Materials and Methods".

An analysis of the residual activity of the fractions exhibiting proteolytic activities with regard to the substrates Z-Val-Lys-Met-MCA and [KMD]APP(-5,+5) was carried out with the aim of continuing the purification of the polypeptides of the invention. At each purification step, the different fractions were first of all brought into contact with the peptide Z-Val-Lys-Met-MCA in order to isolate the fractions which exhibited endoproteolytic activities. These latter fractions were then tested with regard to the [KMD]APP(-5,+5) peptide in order to isolate those fractions which preferentially cleave this peptide substrate at the Met⁻¹-Asp⁺¹ peptide bond (corresponding to the Met⁵⁹⁶-Asp⁵⁹⁷ site in the intact APP). The results of these studies made it possible to identify different fractions which exhibited endoproteolytic activities and which were isolated from supernatants of the 8 cell cultures selected using these two substrates in parallel.

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Several protein fractions were obtained during the last step of the purification process, which is a step of exclusion chromatography on a TSK 2000 column.

Measuring the residual activity of these fractions with regard to the [KMD]APP(-5,+5) peptide made it possible to obtain a single fraction which has an activity of the β -secretase type. This fraction was characterized by measuring its molecular weight by polyacrylamide gel electrophoresis, measuring its isoelectric point, determining its maximum activity as a function of pH, and also determining its profile of inhibition by standard inhibitors ("Materials and Methods").

The electrophoresis analysis was carried out on a Phast-system (Pharmacia) 4-20% polyacrylamide gel under denaturing or normal conditions, and shows a band having a molecular mass in the vicinity of 70 kDa.

The maximum activity with regard to the [KMD]APP(-5,+5) peptide was observed at pH values of between 7 and 8.

The inhibition profile of this fraction with regard to the [KMD]APP(-5,+5) peptide shows that the fraction is a serine protease, with the calculated inhibition percentages being, respectively, 100% in the case of PMSF, 75% in the case of pefablock, 25% in the case of TPCK, 10% in the case of benzamidine and 0% in the case of antipapain.

This example therefore describes the purification process and the search for, and demonstration of, the different characteristics of the polypeptides of the invention possessing a β -secretase activity.

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Example 3. Specificity of the β -secretase activity

This example describes the analysis of the β -secretase activity of the polypeptides of the invention.

This specificity was analyzed using different substrates such as:

- peptides which mimic or duplicate the amino acid sequence of the precursor at the level of the cleavage site and which are described in Table 1.
- the β -amyloid peptide precursor in its natural and mutated (Swedish mutation) forms.

The polypeptides of the invention were brought into contact with the different substrates and the percentage cleavage of these substrates was calculated. The results are presented in Table 3.

In the case of the synthetic peptides, analysis of the findings, which are compiled in Table 3, shows the characteristics relating to the importance of some subsites involved in the recognition of the substrate by this β -secretase and allow it to be concluded that:

1) The subsites P_1 and P_2 are essential (Part A of Table 3), with this being the case whatever the size of the substrates. It was observed that the double mutation (Lys-Met \Rightarrow Asn-Leu) totally abolishes the enzymic cleavage.

2) The subsites P_2 and P_1 are interactive or cooperative (Part B of Table 3). Thus, a single substitution in P_2 (Lys \Rightarrow Asn) or in P_1 (Met \Rightarrow Leu) only decreases the level of cleavage whereas the so-called "Swedish" double mutation abolishes recognition of the substrate.

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Substitution of the residue in P₂ (Lys⇒Arg) finds expression in a difference between the levels of cleavage of the peptides having Leu in P₁ ([KLD]-APP(-5,+5) and [RLD]-APP(-5,+5)) which is greater than that observed in the case of substrates having Met in P₁ ([KMD]-APP(-5,+5) and [RMD]-APP(-5,+5)).

3) The size and/or the volume of the residue in P₁ are important (Part C Tab. 3):

The level of enzymic cleavage decreases when the constraint exercised on the peptide skeleton by the side chain of the P₁ subsite increases. Thus, the experiments which were carried out make it possible to obtain a grading of the level of cleavage in terms of the substitution effected:
[KMD]-APP(-5,+5)>[KLD]-APP(-5,+5)>[KID]-APP(-5,+5)>[KVD]-APP(-5,+5)

4) The residue of the P'₁ subsite is necessarily Asp or Glu (Part D Tab. 3):

Thus, the results demonstrated that the mutation of Asp with Asn or Gln does not abolish cleavage of the substrate; what is more, the cleavage takes place at the Ala-Glu site, equivalent to the Met-Asp site; furthermore, the Ala-Glu pseudosite is only accessible in the natural substrate since, under the same experimental conditions, the level of cleavage of the APP(1,5) fragment is only 35%.

Thus, based on the results which were previously obtained with the polypeptides of the invention with regard to the β-secretase-type cleavage specificity, it is possible to envisage obtaining inhibitors which are of peptide,

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pseudopeptide or non-peptide nature and which are competitive with the Met-Asp cleavage site.

In the case of the β -amyloid peptide precursors of membrane origin, the products of the cleavage of the "normal" (APP-K⁵⁹⁵ M⁵⁹⁶) and "double mutated" (APP-N⁵⁹⁵ L⁵⁹⁶) full-length precursors by the polypeptides, whose β -secretase activity was demonstrated in Examples 1 and 2, were visualized by immunoblotting using the antibodies 22C11 and WO-2.

Analysis of the molecules using these antibodies shows that the percentage cleavage of the APP-KM precursor increases whereas that of the APP-NL precursor remains virtually zero, with this being the case whatever the time of incubation in contact with the enzyme. Thus, the results presented in Figure 3 demonstrate that:

- in the case of the bac.NL membranes, that is to say the incubated membranes containing the APP-NL precursor, the same bands are found whatever the experimental conditions (lanes 1, 2 and 3). Thus, no cleavage by the polypeptides of the invention is observed.

- in the case of the bac.WT membranes, that is to say the incubated membranes containing the natural APP-KM precursor, a new band of about 12 kDa appeared in lane 3 as compared with the other two lanes. Lane 1 depicts the unincubated membranes without enzyme, lane 2 depicts the membranes which were incubated at 37EC without enzyme, while lane 3 corresponds to the membranes which were incubated at 37EC together with the polypeptides of the invention exhibiting an activity of the β -secretase type. It is to be noted that the

difference in the intensity of the bands between lanes 1 and 2 in the case of the bac.WT membranes is due to the quantity of starting material which was loaded onto the gel.

The analysis using the WO-2 antibody revealed this new band, which has a molecular mass of about 12 kDa and which corresponds to the COOH-terminal fragment derived from the precursor being cleaved by the β -secretase at the Met-Asp bond. This analysis permits the conclusion that the natural APP-KM precursor is cleaved by the polypeptides of the invention.

This result indicates, therefore, that the APP-KM precursor, and not the APP-NL precursor, was cleaved selectively, and confirms the findings obtained with the APP substrate peptides of 10, 20 or 40 amino acids in length.

In addition, this example demonstrates that the previously isolated polypeptides of the invention have a β -secretase-type activity which is specific for the natural β -amyloid peptide precursor.

Peptides	Amino acid sequences			
	P ₂	P ₁	P'	
APP(1,+5)			Asp	AEFR
[KMD]-APP(-5,+5)	SEV	Lys Met	Asp	AEFR
[RMD]-APP(-5,+5)	SEV	<u>Arg</u> Met	Asp	AEFR
[KLD]-APP(-5,+5)	SEV	Lys <u>Leu</u>	Asp	AEFR
[RLD]-APP(-5,+5)	SEV	<u>Arg</u> <u>Leu</u>	Asp	AEFR
[NLD]-APP(-5,+5)	SEV	<u>Asn</u> <u>Leu</u>	Asp	AEFR
[NMD]-APP(-5,+5)	SEV	<u>Asn</u> Met	Asp	AEFR
[KID]-APP(-5,+5)	SEV	Lys <u>Ile</u>	Asp	AEFR
[KVD]-APP(-5,+5)	SEV	Lys <u>Val</u>	Asp	AEFR
[KMN]-APP(-5,+5)	SEV	Lys Met	<u>Asn</u>	AEFR
[KMQ]-APP(-5,+5)	SEV	Lys Met	<u>Gln</u>	AEFR
[KM]-APP(-10,+10)	KTEEISEV	Lys Met	Asp	AEFRHDSGY
[NL]-APP(-10,+10)	KTEEISEV	<u>Asn</u> <u>Leu</u>	Asp	AEFRHDSGY
[KL]-APP(-10,+10)	KTEEISEV	Lys <u>Leu</u>	Asp	AEFRHDSGY
[NM]-APP(-10,+10)	KTEEISEV	<u>Asn</u> Met	Asp	AEFRHDSGY
[KM]-APP(-20,+20)	TRPGSLTNIKTEEISEV	Lys Met	Asp	AEFRHDSGYEVHHQKLVFF
[NL]-APP(-20,+20)	TRPGSLTNIKTEEISEV	<u>Asn</u> <u>Leu</u>	Asp	AEFRHDSGYEVHHQKLVFF

Table 1: Table of the peptides of different sizes which mimic or duplicate the β -secretase cleavage site and which were synthesized for being used as substrates in characterizing, and determining the enzyme specificity of, the polypeptide according to the invention.

Inhibitors	Cell lines							
	HMCB	H9	Hs27	IM-9	MRC-5	THP-1	SW1088	SW1783
Standard	100	100	100	100	100	100	100	100
E64 (0.1 mM)	97	87	88	100	87	82	100	92
EDTA (3.3 mM)	<u>43</u>	<u>59</u>	<u>27</u>	<u>71</u>	<u>31</u>	<u>54</u>	<u>39</u>	92
pepstatin (10 μ M)	100	100	88	100	99	100	100	100
chymostatin (5 μ M)	95	87	<u>72</u>	97	<u>75</u>	<u>71</u>	100	100
aprotinin (0.8 μ M)	90	100	<u>40</u>	97	97	93	100	100
pefabloc (3.3 μ M)	<u>53</u>	<u>62</u>	<u>13</u>	97	<u>34</u>	<u>54</u>	91	<u>54</u>
phosphoramidon (70 μ M)	97	<u>62</u>	83	79	<u>66</u>	89	<u>70</u>	<u>76</u>
captopril (60 μ M)	96	87	97	100	100	100	100	100

Table 2: Profile of inhibition of the enzymatic activities of the 8 cell lines selected with respect to the peptide **Z-Val-Lys-Met-MCA**, expressed as a percentage of activity.

	Peptides	Cleavage (%)	Bond cleaved
	(A)-Swedish mutation: effect of size		
	[KMD]-APP(-5,+5)	65	Met [▼] Asp
	[NLD]-APP(-5,+5)	0	
5	[KM]-APP(-10,+10)	45	not
	[NL]-APP(-10,+10)	0	determined
	[KM]-APP(-20,+20)	90	not
	[NL]-APP(-20,+20)	0	determined
10	(B)-Swedish mutation: importance of the P₂ and P₁ subsites		
	[NMD]-APP(-5,+5)	45	Met [▼] Asp
	[KMD]-APP(-5,+5)	65	Met [▼] Asp
	[KLD]-APP(-5,+5)	60	Leu [▼] Asp
	[NLD]-APP(-5,+5)	0	
15	[KMD]-APP(-5,+5)	65	Met [▼] Asp
	[RMD]-APP(-5,+5)	80	Met [▼] Asp
	[KLD]-APP(-6,+5)	60	Leu [▼] Asp
	[RLD]-APP(-5,+5)	20	Leu [▼] Asp
	(C)-Substitution in P₁		
20	[KMD]-APP(-5,+5)	65	Met [▼] Asp
	[KLD]-APP(-5,+5)	60	Leu [▼] Asp
	[KID]-APP(-5,+5)	40	Ile [▼] Asp
	[KVD]-APP(-5,+5)	15	Val [▼] Asp
	(D)-Substitution in P'₁		
25	[KMD]-APP(-5,+5)	65	Met [▼] Asp
	[KMN]-APP(-5,+5)	70	Ala [▼] Glu
	[KMQ]-APP(-5,+5)	80	Ala [▼] Glu
	APP(1,+5)	35	Ala [▼] Glu

Table 3: Results of the analysis of the enzyme specificity of the polypeptide of the invention using

peptides which mimic or duplicate the sequence of the amino acids of the APP precursor at the level of the cleavage site.

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CLAIMS

1. Polypeptide which possesses an activity of the β -secretase type, characterized in that it is able to cleave the natural precursor (APP) of the β -amyloid peptide specifically.
2. Polypeptide according to claim 1, characterized in that the β -amyloid peptide precursor (APP) does not carry any mutation in its protein sequence.
3. Polypeptide according to claim 1 or 2, characterized in that it is a polypeptide which has been purified from human cells from an individual who is not suffering from Alzheimer's disease.
4. Polypeptide according to one of claims 1 to 3, characterized in that it:
 - possesses a molecular mass of about 70 kDa
 - possesses an isoelectric point of about 6.0
 - is an endopeptidase of the serine protease family
 - is an endopeptidase of the chymotrypsin-sensitive type
 - achieves a maximum activity at a pH of between 7 and 8.
5. Polypeptide according to claim 4, characterized in that its activity does not depend on a second substrate and/or ligand.
6. Polypeptide according to claim 5, characterized in that its activity does not depend on ions, preferably calcium or magnesium cations.
7. Non-peptide compound, or compound which is not exclusively peptide in nature, which compound is able to cleave the β -amyloid peptide precursor at the β -secretase site and is obtained by duplicating the active

motifs of the polypeptide according to claims 1 to 6 with non-peptide structures or structures which are not exclusively peptide in nature.

8. Polypeptide according to one of claims 1 to 7, characterized in that it additionally comprises a signal sequence.

9. Polypeptide according to claim 8, characterized in that the signal sequence is selected from the sequence of the signal peptide of IgkB, the signal peptide of APP and the signal peptides of the subunits of the muscle and central nervous system nicotinic acetylcholine receptors.

10. Process for purifying, from cells derived from individuals who are not suffering from Alzheimer's disease, a polypeptide according to one of claims 1 to 9, characterized in that the following steps are carried out:

- the supernatant from the cell culture is removed and then concentrated
- the concentration product is once again concentrated on a tangential membrane
- the resulting product is then purified by means of consecutive steps of chromatography, in particular by means of steps of exclusion chromatography, ion exchange chromatography and hydrophobic interaction chromatography.

11. Use of a human cell line, which represents the central or peripheral nervous system and the immune system and which is able to carry out the normal metabolism of the β -amyloid peptide precursor, for producing the polypeptides of the invention which are defined in accordance with claims 1 to 9.

b - the molecules which are bound to the said polypeptide are detected and/or isolated.

15. Ligand for a polypeptide as defined according to claims 1 to 9, which can be obtained according to the process of claim 14.

16. Ligand according to claim 15, characterized in that it is an antagonist, an agonist or an inhibitor of the polypeptide defined according to claims 1 to 9.

17. Pharmaceutical composition which comprises, as the active principle, at least one inhibitor of the polypeptide according to one of claims 1 to 9.

18. Pharmaceutical composition which comprises, as the active principle, at least one antibody or antibody fragment according to claim 13 and/or one ligand according to claim 15.

19. Pharmaceutical compositions in which the peptides, antibodies or antibody fragment according to claim 13, and ligands and/or corresponding nucleotide sequences defined according to claim 15 are combined with each other or with other active principles.

20. Composition according to one of claims 17 to 19 which is intended for at least partially inhibiting the interaction between the polypeptide and the β -amyloid peptide precursor and/or inhibiting the activity of the polypeptide.

21. Composition according to one of claims 17 to 20 which is intended for intervening in the metabolism of the β -amyloid peptide and, preferably, for inhibiting or retarding production of the β -amyloid peptide.

22. Composition according to one of claims 17 to 21 which is intended for treating neurodegenerative diseases.

23. Composition according to claim 22 which is intended for treating Alzheimer's disease.

24. Use of an antibody or antibody fragment according to claim 13 and/or a ligand according to claim 15 for at least partially inhibiting the interaction between the polypeptide and the β -amyloid peptide precursor and/or inhibiting the activity of the polypeptide and/or intervening in the metabolism of the β -amyloid peptide.

25. Use of an antibody or antibody fragment according to claim 13 and/or a ligand according to claim 15, as a medicinal product, especially for treating neurodegenerative diseases and in particular Alzheimer's disease.

26. Use of the polypeptides according to claims 1 to 9 for preparing a medicament intended for treating neurodegenerative diseases, in particular Alzheimer's disease.

27. Use of the polypeptides according to claims 1 to 9 for detecting ligands of the polypeptides and/or compounds which are able to at least partially inhibit the interaction between the polypeptide and the β -amyloid peptide precursor and/or inhibit the activity of the polypeptide and/or intervene in the metabolism of the β -amyloid peptide.

28. Method for detecting molecules which modify the activity of the polypeptides of the invention, which method comprises the following steps:

- the polypeptides of the invention which exhibits an activity of the β -secretase type are brought into contact with a molecule or a mixture which contains different molecules, which may not have been identified,

- the reaction mixture described in the preceding step is brought into contact with the substrate of the polypeptides of the invention, which substrate is preferably APP in its natural form

- the β -secretase activity on the APP is measured
- the molecules which modify the β -secretase activity of the polypeptides of the invention are detected and/or isolated.

29. Viral or plasmid vector which contains the nucleotide sequences of the molecules which are agonists or antagonists of the polypeptides of the invention, for transfecting the said sequences into appropriate host cells and expressing the said molecules which are agonists or antagonists of the polypeptides of the invention in vivo, ex-vivo and/or in vitro.

The invention concerns novel peptides and their pharmaceutical use. More particularly, the invention concerns novel polypeptides having a β -secretase type activity characterized in that they are capable of specifically cleaving the natural β -amyloid peptides precursor (APP).

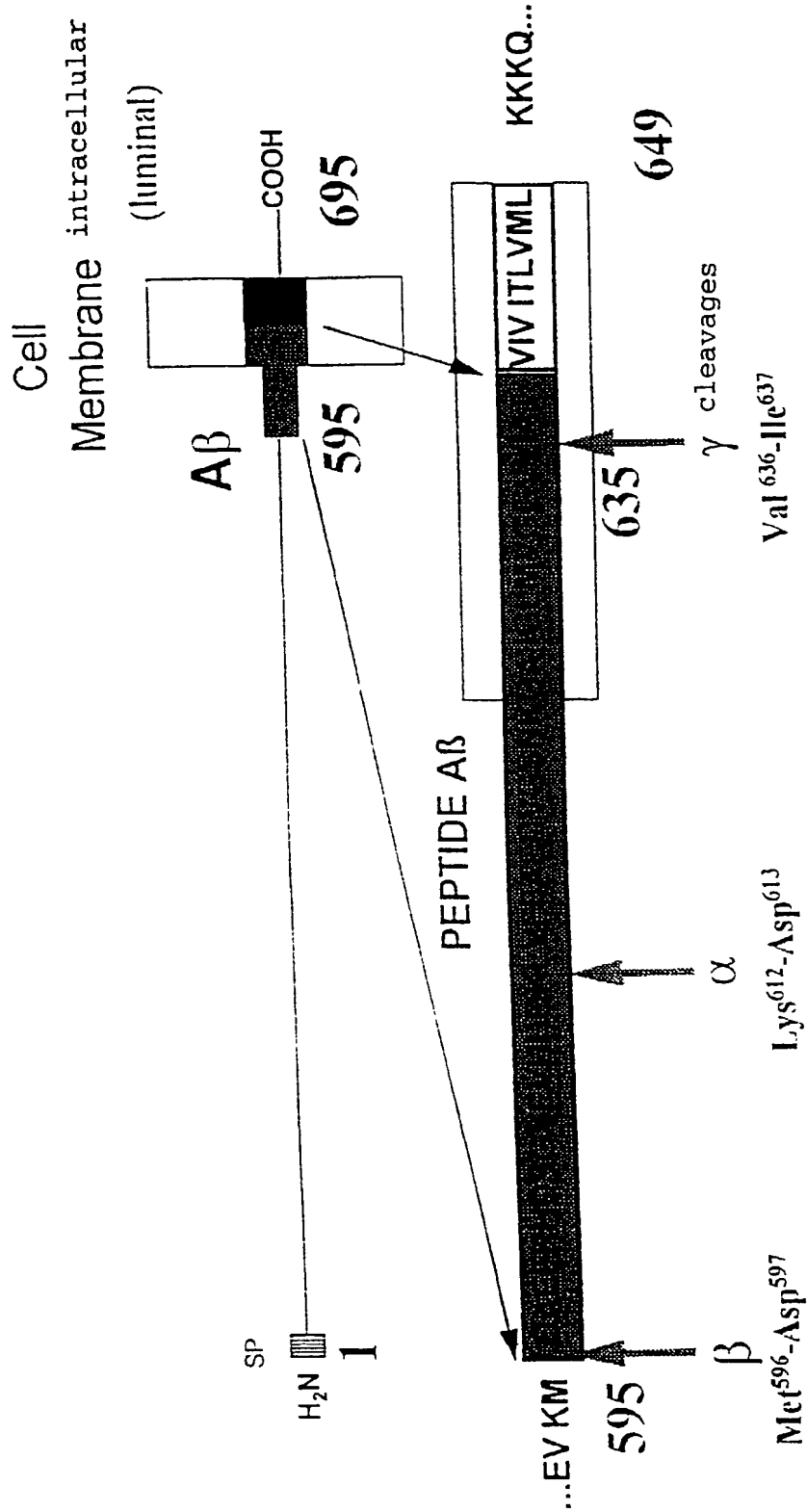
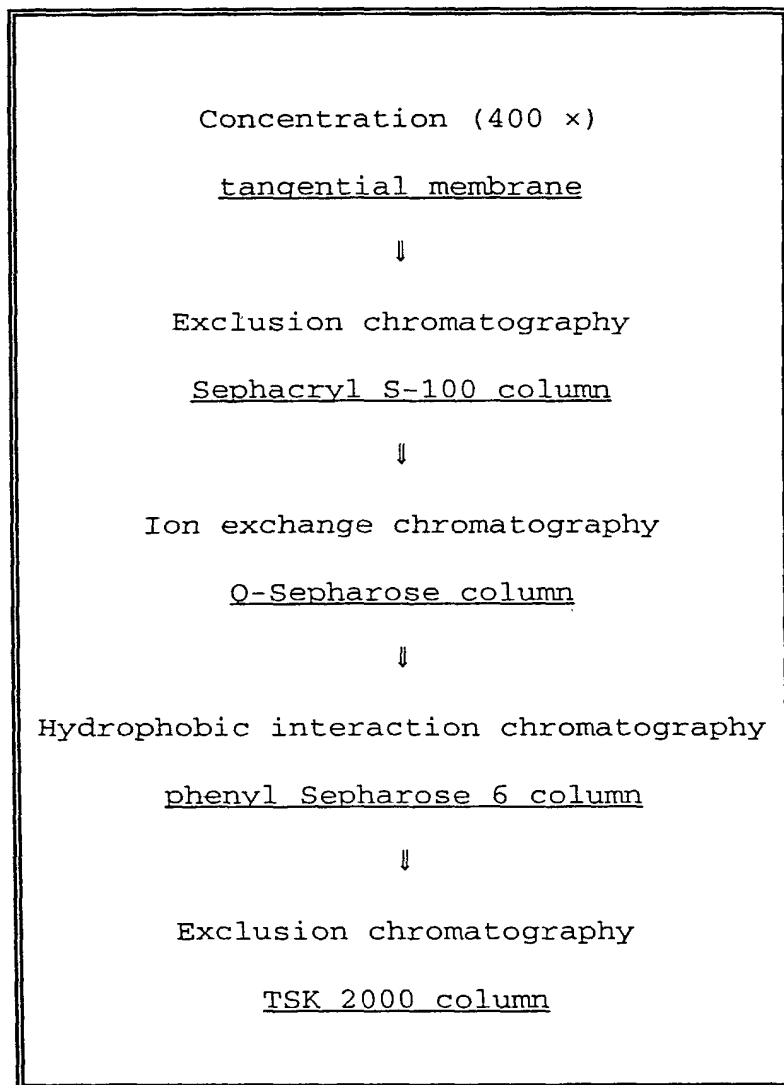


Figure 1

**Figure 2**

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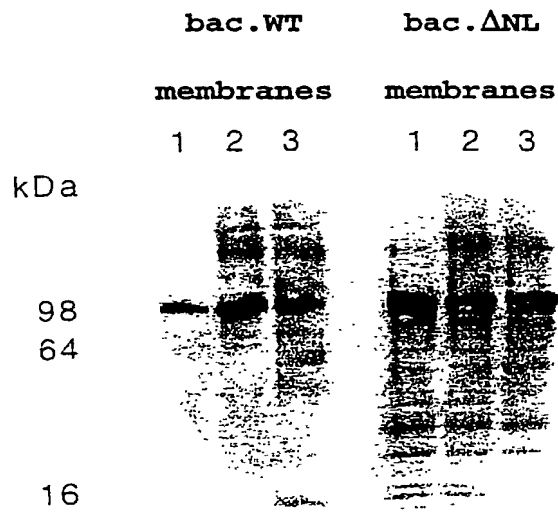


Figure 3

DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION

UNITED STATES OF AMERICA

As a below named inventor(s), I/We hereby declare that:

My/Our residence(s), post office address(es) and citizenship(s) are as stated below my name(s).
I/We verily believe I am/we are the original, first and sole/joint inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

POLYPEPTIDES WHICH POSSESS AN ACTIVITY OF THE B-SECRETASE TYPE

and the specification of which ☐ is attached hereto (Attorney Docket No. **ST98014A-US**)
(check one) ☒ was filed on **4 January 1999** as U.S. Application Number **09/701,945**
and was amended on (if applicable).
☐ was described and claimed in PCT Int'l Application Number filed on
and as amended under PCT Article 19 on (if any).

I/We hereby state that I/We have reviewed and understand the contents of the above identified specification, including the claims as amended by any amendment referred to above.
I/We acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 C.F.R. 1.56.

☒ I/We hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or 365 (b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one other country other than the United States of America, listed below and having a filing date before that of the application on which priority is claimed. I/We have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Foreign Priority:	<u>98 07068</u> Number	<u>France</u> Country	<u>5 June 1998</u> Day/Month/Year Filed
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Prior Foreign Appln(s):	<u> </u> Number	<u> </u> Country	<u> </u> Day/Month/Year Filed
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☒ I/We hereby claim the benefit under Title 35, United States Code §119(e) of any United States Provisional application(s) listed below:

<u>60/122,599</u> Number	<u>3 March 1999</u> Filing Date
-----------------------------	------------------------------------

☒ I/We hereby claim the benefit under Title 35, United States Code §120 or 365(c) of any United States application(s) or international application designating the United States listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I/We acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

<u>PCT/FR99/01326</u> Application Serial No.	<u>4 June 1999</u> Filing Date	<u>Completed</u> Status (Patented, Pending)
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I/We hereby appoint the attorneys and/or agents associated with the Customer No.(s) provided below as my/our attorneys and/or agents with full power to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Customer No.: 005487

I/We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States code §1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

FOR PATENT APPLICATION

005487-005487

below named inventor(s), I/We hereby declare that:

My/Our residence(s), post office address(es) and citizenship(s) are as stated below my name(s).
I/We verily believe I am/we are the original, first and sole/joint inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Polypeptides Which Possess An Activity Of The β -Secretase Type

and the specification of which ☒ is attached hereto (Attorney Docket No. **ST98014A**)
(check one) ☐ was filed on _____ as U.S. Application Number _____
and was amended on _____ (if applicable).
☐ was described and claimed in PCT Int'l Application Number _____ filed on _____
and as amended under PCT Article 19 on _____ (if any).

I/We hereby state that I/We have reviewed and understand the contents of the above identified specification, including the claims as amended by any amendment referred to above.
I/We acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 C.F.R. 1.56.

☒ I/We hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or 365 (b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one other country other than the United States of America, listed below and having a filing date before that of the application on which priority is claimed. I/We have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Foreign Priority: Number _____ Country _____ Day/Month/Year Filed _____

Prior Foreign Appln(s): **PCT/FR99/01326** **PCT** **04 June 1999**
Number Country Day/Month/Year Filed

☒ I/We hereby claim the benefit under Title 35, United States Code §119(e) of any United States Provisional application(s) listed below:

60/122,599 **03 March 1999**
Number Filing Date

☐ I/We hereby claim the benefit under Title 35, United States Code §120 or 365(c) of any United States application(s) or international application designating the United States listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I/We acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

Application Serial No. _____ Filing Date _____ Status (Patented, Pending) _____

I/We hereby appoint the attorneys and/or agents associated with the Customer No.(s) provided below as my/our attorneys and/or agents with full power to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Customer No.: 005487

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I/We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States code §1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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